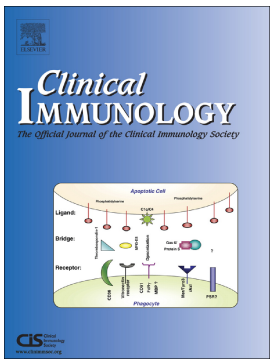

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Article

Recombinant human granulocyte macrophage-colony stimulating factor expressed in yeast (sargramostim): a potential ally to combat serious infections

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Abstract:

Granulocyte-macrophage-colony stimulating factor (GM-CSF), can direct the activation, proliferation and differentiation of myeloid-derived cells. It is also responsible for maturation and function of professional antigen presenting cells thereby impacting adaptive immune responses, while assisting to maintain epithelial barrier function. GM-CSF in combination with other endogenous cytokines and secondary stimuli, such as tumor necrosis factor can modulate pro-inflammatory monocyte priming via chromatin remodeling and enhanced transcriptional responses, a concept termed “trained immunity”. An increase in the incidence of opportunistic fungal infections was recently reported in patients with hematological cancers receiving treatment with the BTK inhibitor, Ibrutinib. Tec Kinase BTK is known to influence the expression of GM-CSFRα and regulates downstream signaling pathways, suggesting a role for GM-CSF in maintenance of defense against fungal infections in immune competent hosts. Further examination of the potential mechanism(s) of action for naturally occurring GM-CSF and recombinant human GM-CSF (rhu-GM-CSF) expressed in yeast (sargramostim) are reviewed.

Introduction

Granulocyte-macrophage colony-stimulating factor (GM-CSF), also known as colony stimulating factor number 2 (CSF-2), was the first member of the ß common chain cytokine family to be identified. It was initially detected in mouse lung-conditioned medium [1] and subsequently described as a hemopoietic cytokine able to differentiate *in vitro* myeloid precursor cells into macrophages and granulocytes from granulocyte-monocyte progenitor (GMP) cells[2-5]. The GM-CSF gene is located on chromosome region 5q31, clustered with other genes encoding Interleukins (IL)-IL-5, IL-4, and IL-3 [6-9]. Collectively, IL-3, IL-5, and GM-CSF can synergize a differentiation and function of myeloid cells as well as coordinating immune responses.

After transcription and transduction, the resulting protein, is glycosylated and secreted into the extracellular environment as a homodimer [10]. Due to the deep conservation of this molecule in the mammalian lineage, GM-CSF cellular sources in the body are multiple. Cellular sources of GM-CSF include T and B cells, such as the innate response activator B cells that reside in the pleural cavity and protect against pneumonia through a GM-CSF-IgM axis [11]. Myeloid cells, such as eosinophils, basophils, mast cells, neutrophils, monocytes, and macrophages produce GM-CSF as well. Tissue-resident cells, such as microglia, endothelial cells, chondrocytes, osteoclasts, fibroblasts, pulmonary epithelial cells and uterine cells can also produce GM-CSF [12]. Furthermore, some tumors have been described as capable of producing GM-CSF [12].

GM-CSF binds only one high affinity receptor; GM-CSFR [13]. The receptor is a heterodimer, composed of an α (GMRα) and β chain (GMRβ) ([14-16]), with the β chain being common to the receptors for IL-3 and IL-5 (IL-3R and IL-5R, respectively) [17,18].

The gene encoding the α chain subunit, CSF2RA, is located in the pseudoautosomal region 1 (PAR-1) of both sexual chromosomes, whilst the gene encoding the β chain is located in chromosome region 22q12.3 [13]. The GM-CSF/GMCSFR receptor-ligand

complex is found in nature as a dodecamer, as recently demonstrated by crystallographic studies [19-21]. As the complex forms, GM-CSF initially binds GMRα [22], then, the heterodimer recruits GMRβ, further strengthening the bond with the ligand and leading to receptor activation [16]. The heterotrimer, composed by GM-CSF+GMRα+GMRβ, then polymerizes with another heterotrimer to form a hexamer (**Figure 1**), which then binds another hexomer to form the dodecamer receptor-ligand active complex [13].

This dodecameric form allows the cytoplasmic tails of two GMRβ, coupled with the Janus kinase 2 (JAK2), to face and self-trans-phosphorylate each other [19]. This event causes the activation of two pathways important for proliferation events, JAK2/STAT5 and MAPK, as well as activation of PI3K/Akt pathways thereby facilitating cell survival by inhibiting, via Mcl-1, the Bax/Bak-related apoptosis induction pathway [13]. Furthermore, both MAPK (by repressing Bim) and IKK (by repressing Puma) contribute to inhibition of Bax/Bak mediated apoptosis [23].

In contrast to GMRβ-related signaling pathways, GMRα-related signaling remains less understood; although signaling pathways such as SLAP, p85, IκKβ, GRAP, Lyn and Src have been implicated, the details of how these pathways signal in combination with GMRα remains incompletely characterized [23]. Other various signaling pathways are also modulated by the extracellular portion of GMRβ that variously interacts with a wide spectrum of molecules including integrin β1, CBAP and FcRγ [23]. GM-CSFR is predominantly expressed by dendritic cells (DCs), granulocytes and eosinophils [24]. The regulation of GM-CSF mRNA remains obscure, however Sturrock and colleagues described a negative effect of miR133a and miR133b on GM-CSF through interaction with the 3' untranslated region (UTR) of GM-CSF [25].

Although, GM-CSF in the literature is described to mediate multiple crucial host response functions to external stimuli such as inflammation and the antitumor response,

the present review aims to focus only on the physiological aspects of GM-CSF and its' role during infection through effects on the properties and functional status of immature and mature myeloid cells.

Recombinant GM-CSF:

Recombinant expressed in yeast GM-CSF (sargramostim) was approved in 1991 for the treatment of neutropenia associated with stem cell transplant and to treat several other causes of neutropenia resulting from leukemia or its treatment. In 2018 the FDA approved sargramostim to increase survival in adult and pediatric patients acutely exposed to myelosuppressive doses of radiation (Hematopoietic Syndrome of Acute Radiation Syndrome, or H-ARS) [26].

GM-CSF and Myelopoiesis

GM-CSF is not essential for normal hematopoiesis, however it is essential for emergency hematopoiesis when there is an increased demand for granulocytes and macrophages to fight infection. A concentration-dependent behavior of GM-CSF induced differentiation of granulocyte-monocyte progenitor (GMP) cells is essential for human health.

Critical proteins for granulocytic commitment include CCAAT enhancer-binding proteins (C/EBPα and C/EBPβ) whose functions are redundant in hematopoiesis and are here referred to collectively as C/EBP, growth-factor independent-1 protein (Gfi-1), GM-CSF receptor (GM-CSFR), and G-CSF receptor (G-CSFR). Weston et. al., performed a mathematical analysis of cytokine-induced differentiation of GMPs and described how, with high concentrations of GM-CSF, C/EBP increases quickly, resulting in a swift rise in Gfi-1 and repression of PU.1, thereby inducing granulopoiesis [6]. This is in accordance with an earlier study by Wang et. al., which showed that C/EBP is an antagonist of PU.1 in granulopoiesis [27].

They also describe a positive feedback loop of GM-CSFR, C/EBP and PU.1 that creates a sensitive, switch like response of monocytic gene expression to GM-CSF stimulation where the lower the concentration of GM-CSF, the longer it will take for the switch to "kick in" shifting the process to monopoiesis. They propose that this delay permits PU.1 to establish dominance over pro-granulocytic transcription factors. Once the cells are committed to monocytic fate, GM-CSFR levels are then upregulated to high levels, resulting in greater GM-CSF signal strength in monocytes.

The Weston model agrees with experimental data from Lee and associates whose model suggests that the GM-CSF signal strength is stronger in the initial commitment step of granulopoiesis and this high signal strength decreases and eventually stabilizes due to GM-CSFR down regulation post-granulocytic commitment. However, in monopoiesis which initiates at low concentrations of GM-CSF, by the time GM-CSF signal is strong, the cell is conclusively committed to that lineage. Therefore, once established into monocytic fate, the capacity to process a strong GM-CSF signal via upregulated GM-CSFR may be important for gene regulation within the monocytes [28,29].

Interestingly it appears that GM-CSF induced granulopoiesis exhibits a larger spike in PU.1 and IRF8 concentrations in its early stages than M-CSF and G-CSF induced granulopoiesis. These differences may affect downstream transcription factors and prime the cells for different subtypes of granulocytes [6]. This may provide a more functional emergency myeloid cellular response to a myelopoiesis crisis.

During infection, inflammation, and cancer, a population of Myeloid-derived suppressor cells (MDSCs) expands with the ability to suppress T-cell responses [30]. Parmiani and associates suggested that monocytes can morph into a Monocytic-MDSC as a consequence of "too high a dose" of exogenous administration of GM-CSF [31]. Weston et. al., postulates that high GM-CSF concentrations can induce a monocyte to transition into an M-MDSC (Figure 2). They postulate that this behavior is a consequence of high

expression of GM-CSFR on committed monocytes that can now translate exogenous increases in GM-CSF concentration to induce transformation into MDSCs via simultaneous upregulation of PU.1 and C/EBP in these cells. Additionally, their results suggest that the stability of this MDSC status is dependent and regulatable via extracellular GM-CSF stimulation.

GM-CSF and immune cells

The immunomodulatory properties of GM-CSF may be exerted ***directly*** on cells of the immune system (**Figure 3**) or ***indirectly*** to target tissues such as lung epithelial cells, uterine cells, fibroblasts and endothelial cells that express GM-CSFRα [32]. The significance of GM-CSF on cells of the immune system is typified by human cytomegalovirus. Once ingested by monocytes, the generated dendritic cells (CMV-MoDCs) acquire a dysfunctional phenotype disrupting not only GM-CSF signaling in the infected MoDCs but also through a paracrine fashion on other DCs [33]. In addition to viruses, *Cryptococcus neoformans* downregulates the production of GM-CSF and TNF-α by unstimulated human NK cells, as assessed by gene expression and supernatant protein levels [34]. Immune evasion circumnavigating GM-CSF by *Candida albicans* (*C.albicans*) has been demonstrated. *C.albicans* impedes alveolar macrophage reactive oxygen species (ROS) production by targeting NADPH oxidase, a major oxidative stress-dependent NF-κB signaling pathway, a central regulator of GM-CSF release following TLR2/6 stimulation of endothelial cells [35]. This NADPH oxidase–dependent regulation of GM-CSF plays an important dual role; 1. in patrolling immune cells for pathogen killing, and; 2) in endothelial cells, where ROS formation controls the release of immunologically relevant growth factors to recruit and differentiate immune cells toward the required effector function [36].

Neutrophils

Neutrophils are sophisticated immune cells that can release specific granular enzymes, immunomodulatory cytokines and chemokines that interact with various components of the immune system.

Although GM-CSF has been postulated to collaborate as a pro-differentiation factor for neutrophils, GM-CSF knock-out mice exhibited no neutropenia [37]. Physiologically the pro-differentiating signal of GM-CSF on myeloid precursors seems to be negligible, as differentiation is believed to be achieved through G-CSF [38]. In contrast, during physiologic challenges, GM-CSF becomes relevant by inhibiting neutrophil migration [39], increasing their lifespan [40], facilitating degranulation [41], increasing IL-1 production [42], and modifying surface reactivity by polarizing the arachidonic metabolism during leukotriene production [43]. The anti-microbial activity of neutrophils is sustained by Natural Killer (NK) cell derived GM-CSF [44]. GM-CSF has also been shown to synergistically activate neutrophil antibody-dependent cellular cytotoxicity (ADCC) [45].

Environmental cues encourage neutrophils to sequester pathogens *via* phagocytosis or by releasing neutrophil extracellular traps (NETs) outside the cell. *Candida albicans*, induces NET formation and both yeast-form and hyphal cells is susceptible to NET-mediated killing, potentially circumventing the need for phagocytosis. This form of NET formation known as vital NETosis is associated with release of mitochondrial DNA that is dependent on myeloperoxidase (MPO) and produced after stimulation with GM-CSF[46].

Interestingly a study of children affected by severe congenital neutropenia (SCN) demonstrated that phagocytosis is apparently normal in SCN neutrophils, suggesting that the reduced killing ability of these cells does not rely on defects of microbial internalization. These children remained at a consistent high risk of infection even under Granulocyte Colony Stimulating Factor (G-CSF) treatment. While G-CSF is capable of correcting neutropenia, the authors conclude that G-CSF is not sufficient to correct all of the functional deficiencies of neutrophils[47]. Recently Khandagale et. al., observed that GM-

CSF restored MPO expression in NETs as well as up-regulated the expression of calprotectin in NETs of a patient with SCN and enabled efficient killing of *C. albicans* yeast and hyphae [48].

Basophils

GM-CSF synergistically acts with IL-3 to induce basophil differentiation in the bone marrow [49]. These differentiated cells, through the expression of MHC-II and other co-stimulatory molecules, promote a Th-2 mediated response *in vivo* [50] and *in vitro* [51].

Eosinophils

GM-CSF exhibits a strong influence on eosinophil lifespan and response to environmental triggers [24,52]. It increases eosinophil mobilization through chemoattractant capacities and optimizes phagocytic and de-granulating capabilities [53].

Mast Cells

Mast cells are key players in allergic and anaphylactic reactions; they also participate in acquired and innate host immune response. Mast cells can express receptors for IL-3, IL-5 and GM-CSF [54] and are thus likely to be activated following exposure to rGM-CSF [55]. Common side effects of high dose rGM-CSF, likely due to off-target effects on IL-3 and IL-5 common beta chain binding include flushing, induction of fever with flu-like symptoms, musculoskeletal pain and hypotension as well as nausea and capillary leak syndrome [56-58] and thus careful dosing of rGM-CSF is warranted.

Monocytes and Macrophages

The role of GM-CSF in DC development appears to be situationally-as well as subset-specific. During bone marrow myelopoiesis, in a steady state condition, macrophages and dendritic precursors (MDPs) differentiate into Common Dendritic precursors (CDPs) and further into pre-committed DCs (pre-DCs) via FMS-like tyrosine kinase 3 ligand (FLT3L) [59]. At physiologically relevant levels of GM-CSF, stimulation of pre-DCs elicits migration into the lymphatic tissue or even peripheral blood where GM-CSF

and other specific tissue-related molecules orchestrate their differentiation into committed DCs, namely CD 103+/CD 8α+/XCR1+/Clec9a+ (Group 1), These Myeloid cDC1 have been characterized as a subset of DC that have a high intrinsic capacity to cross-present antigens via MHC class I to activate CD8+ T cells and to promote T helper type 1 (Th1) and natural killer responses through IL-12. Differentiation may alternatively lead towards CD 11b+/CD4+/ESAM+ or neg (Group 2 cDCs) [59].

During inflammation, activated CD4+ T cells produce large amounts of GM-CSF that act in an endocrine manner driving the so-called "emergency myelopoiesis" [60]. Macrophage dendritic cell precursors (MDPs) differentiate into committed monocyte precursors (cMoPs) that further differentiate in the bone marrow into Ly-6C^{hi} monocytes in mice. Following a GM-CSF gradient Ly-6C^{hi} monocytes extravasate and further differentiate into inflammatory macrophages (F4/80^{lo}CD11b^{hi}) which release high levels of IL-1 and IL-6, or, in the presence of IL-4, differentiate into monocytic functional DCs (CD64^{+}CD11b^{hi} , MoDCs), or short-lived Langerhans cells [60].

Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by GM-CSF plus interleukin 4 and downregulated by tumor necrosis factor-α [61].

Macrophages and host defense

Macrophages play a central role in host defense. They are capable of engulfing (phagocytosing) invading organisms following recognition through pattern recognition receptors (PRRs) of pathogen-associated molecular patterns (PAMPs) as well as innate host damage-associated molecular patterns (DAMPs) which are upregulated following microbial invasion or cellular damage.

Global gene expression analyses of macrophages differentiated from GM-CSF-treated monocytes has demonstrated GM-CSF upregulation of 340 genes and downregulation of 190 genes in macrophages. Macrophage-specific genes including

CD14, CD163, C5R1, and FcgR1A, several cell surface adhesion molecules, and cytokine receptors are induced by GM-CSF [62].

The effects of GM-CSF on macrophage function is extensive and includes; enhancing macrophage antigen presentation capacity and antibody mediated phagocytosis via complement.[54] Macrophage microbicidal capacity, leukocyte chemotaxis and adhesion are also enhanced by GM-CSF. GM-CSF also induces the production of cytokines IL-6, IL-8, G-CSF, M-CSF, TNF and IL-1 [63].

In response to host challenge, macrophages may induce both pro- and anti-inflammatory pathways based upon contextual signaling. Classical or "M1" type macrophages, in response to IFNγ signaling, produce pro-inflammatory cytokines, upregulate MHCII antigens and increase inducible nitric oxide synthase (iNOS) production and reactive oxygen intermediates. M1 macrophages promote Th1 response and possess strong microbicidal and tumoricidal activity. Conversely, following IL-4/IL-13 stimulation, alternative or "M2" macrophages produce arginase, increased IL-10 levels, upregulate CD206 and increase polyamines to stimulate cell growth and repair. M2 macrophages promote a Th2 response, tissue remodeling, immune tolerance and tumor progression.

However, the concept of M1 vs. M2 macrophages has been demonstrated to be a vast oversimplification of the role and lineage of macrophages. For instance, recent elegant studies have demonstrated that tissue macrophages have a dual ontogeny and can develop from circulating monocytes that enter the tissue, as well as from embryonic precursors derived from the yolk sac. Thus, replenishment of tissue macrophages varies based upon the type of challenges encountered as well as anatomical site(s).

This wide range of response and derivation of macrophages has solidified the contention that macrophages represent a highly heterogeneous population with high levels of plasticity that rely on contextual signaling to stimulate the correct response. Thus,

macrophages can coordinate inflammatory responses, including initiation as well as termination of inflammation.

GM-CSF in combination with other endogenous cytokines has been demonstrated to modulate the pro-inflammatory activation of CD14+ monocytes through secondary stimuli (e.g., TNFα), resulting in a primed monocyte that is likely to recognize secondary challenges (i.e., subsequent infection) more efficiently due to induced chromatin remodeling and enhanced transcriptional response, a relatively new concept termed trained immunity. The ability of pathogens, such as fungi to induce trained immunity is a recently emerging concept that expands the repertoire of host innate response [64]. The prime example of this phenomena is shown in fungal infections. Quintin et al demonstrated that exposure of mice to a low dose (non-lethal) of *C. albicans*, 7 days prior to a toxic challenge dose afforded protection of wild type and Rag $1^{-/-}$ mice deficient in T/B cells. This protection was not shown in animals with a compromised monocyte migratory signal system ($Ccr2^{-/-}$). The authors demonstrate that C*. albicans* triggers an initial protective response that can augment the inflammatory response through recognition of Candidal beta glucans and induction of epigenetic changes in monocytes via dectin-1/Raf-1 signaling [65]. This result is postulated to play a contributory role in the commensal niche occupied by *Candida* in the human host [66].

There is a balance between metabolism and macrophage cell function mediated through GM-CSF that results in the induction of inflammatory macrophages via changes in cellular metabolism. GM-CSF–dependent macrophage functions require mTOR/Akt/ERK signaling–mediated *de novo* synthesis of c-myc. This signaling pathway results in glucose transporter (GLUT) upregulation and increased basal glucose uptake. TLR activation–mediated acute glycolysis is robustly induced in macrophages primed by GM-CSF, and the consequent mevalonate pathway activation acts as a bridge between glycolytic capacity and inflammatory phenotypes. Na et. al., demonstrated that c-myc is a major transcription

factor in GM-CSF–mediated inflammatory polarization upon physiologic stimulation. This is meaningful because, several published studies have reported c-myc as a typical central regulator of glycolysis metabolism in cancer cells as well as T cells [67].

GM-CSF and bacterial infection:

GM-CSF plays a pivotal role during bacterial infections. The prototypical example of GM-CSF function in infection can be seen in pneumonia patients. Bacteria, via TLR-4 [68], and viruses, via HGF/c-MET and TGF-α/EGFR [69], can activate alveolar macrophages. GM-CSF is produced in low physiological quantities by the respiratory epithelium to recruit, in a paracrine manner, alveolar macrophages to clean the surfactant deposits preventing Pulmonary Alveolar Proteinosis (PAP) [70]. Pulmonary alveolar proteinosis (PAP) is a severe autoimmune disorder that results from autoantibodies-induced to neutralize GM-CSF- inhibiting alveolar macrophage function in an off-target effect [71]. In addition, mutations in or development of antibodies to GM-CSF or GM-CSFR has also been associated with nocardia infections [72]. During infection respiratory epithelia begin to produce robust amounts of GM-CSF that recruits and activates the *resident* CD103+ monocytes, an indispensable subset for CD8+ T clearance [73]. Epithelial GM-CSF production is mainly driven by activated alveolar macrophages in a TNF-dependent manner [74]. At the same time, in the early infection phase, B1a serosal B cells (CD43+CD5+) activate and produce large amounts of GM-CSF that amplify the cellular interaction and furthermore, via CD131, stimulate the production of neutralizing IgM in an autocrine manner [11]. Remarkably, low level epithelial-derived GM-CSF triggered by alveolar macrophage interaction may also contribute to the resolution of inflammation and tissue repair by recruiting type II alveolar epithelial cells (AEC II) [74].

GM-CSF produced by activated T cells (CD4+, CD8+ T cells as well as non-conventional iNKT cells and γδ T cells) following challenge with *Mycobacterium*

tuberculosis (MTB), the causative agent of tuberculosis (TB), has been demonstrated to mediate host protection [75]. In addition, recruitment of leukocytes following MTB infection leads to the upregulation and expression of cytokines from infected macrophages. GM-CSF has been proposed to direct the polarization of macrophages toward the M1 phenotype through upregulation of proinflammatory cytokines including IL-6, IL-8, IL-1 and TNFα. Thus, GM-CSF upregulation following MTB infection leads to a proinflammatory response that augments host innate response to MTB. In a study by Pedral-Sampaio and associates, evaluating the safety of sargramostim as adjuvant immunotherapy for the treatment of active human pulmonary tuberculosis, a trend toward faster conversion to negative culture was observed in the sargramostim group [76]. Thus, augmenting GM-CSF response following MTB infection may enhance host immunity. This may be an especially important concept given the evolving number of resistant MTB strains that have recently emerged.

Bermudez and colleagues demonstrated that sargramostim induced micro-bactericidal and -static activity in macrophages *in vitro* and *in vivo*; the combination of sargramostim and amikacin (50 mg/kg) or azithromycin (250 mg/kg) was associated with a significant increase in killing of *Mycobacterium avium* complex both within cultured macrophages and in the beige mouse model. Therefore, a significant reduction in the number of viable bacteria was observed in the blood, liver, and spleen of mice treated with a combination of sargramostim and azithromycin or amikacin compared with control mice and those treated with sargramostim or antimicrobials alone [77].

GM-CSF and viral infection:

Influenza virus, a leading cause of acute respiratory tract disease, also infects AEC II, the main cell type of the alveoli in charge of gas-exchange. AEC II cells produce high levels of GM-CSF during viral infections upon encountering HGF and TGF-α. Interestingly,

both stimulations may act in either an autocrine and/or paracrine fashion; in fact, only AEC II cells possess c-MET, the HGF receptor, EGFR, and the TGF-α receptor. TGF-α is expressed by AEC II cells and by neutrophils, whilst HGF is expressed only by neutrophils [69]. This differential expression of GM-CSF stimulatory molecules allows AEC II cells to exhibit fine modulation through concentration-dependent effects of GM-CSF, resulting in activation of innate and adaptive immunity, resulting in improved viral clearance.

In the lung, GM-CSF can direct the activation, proliferation and differentiation of myeloid-derived cells. These cells participate in the immune response designed to protect the lung from challenges such as those encountered following influenza infection. In addition to the respiratory challenge of the virus itself, a major health threat associated with influenza is complications caused by secondary bacterial infections that frequently accompany influenza. GM-CSF promotes alveolar macrophage maturation and antimicrobial function, an effect that is dependent on the transcription factor PU.1 [78]. Increased levels of GM-CSF in the lung alveolar macrophage lead to increased production of reactive oxygen species (ROS) that is protective from viral, as well as secondary bacterial infections. GM-CSF–driven DC expansion during pulmonary infections results in the generation of DCs with robust immune function that are prone to the induction of Th1 immune responses against these infections [79,80]. Halstead and associates have established a mouse therapeutic model of GM-CSF, wherein GM-CSF is “administered” to the airways 3 days after establishment of infection and confers protection. Their work suggest that high levels of GM-CSF drive the classically activated M1-like monocytes/macrophages in the lung during Influenza A viral infection towards an M2-like phenotype [81]. Previously Cole and associates established that an elevated M1/M2 macrophage ratio in the lungs contributes to disease severity [82]. Halstead’s group demonstrates that *in vivo,* high airway levels of GM-CSF markedly rescue mice from lethal influenza pneumonia. This is of key concern as pulmonary infections such as influenza

induce lung injury and disrupt the barrier integrity of lung vasculature and results in increased albumin levels in the alveolar space [83].

GM-CSF has also been demonstrated to protect the lung by restoring barrier function and stimulating epithelial cell proliferation [83]. GM-CSF exerts a protective effect on the alveolar epithelium against oxidative stress-induced mitochondrial injury [84].

Thus, augmentation with sargramostim through stimulation of innate immune responses may enhance protection against viral and potential secondary infections that could compromise the alveolar epithelium. Sargramostim may become an important treatment strategy for management of pulmonary infections, particularly in the elderly, the very young and against drug-resistant strains.

GM-CSF and fungi:

A major concern in the clinical setting of the intensive care unit and in patients with mechanical ventilation arises from a pathologic mycobiome, *C. albicans,* in which airway colonization may facilitate the development of not just *Pseudomonas aeruginosa* (*P. aeruginosa)* but also *Staphylococcus aureus* (*S.aureus*) as well as *Escherichia coli* (*E. coli*).

The role of GM-CSF in fungal infection is complex. We hypothesize that GM-CSF acts by potentiating calcineurin, Bruton's tyrosine kinase (BTK) and CARD9 antifungal pathways via signal convergence. GMR-α signals may converge via MAPK and PI3K/AKT signals. MAPK signals converge on IKK leading to decreased apoptosis signaling and increased lifespan, as observed in neutrophils [85]. The IKK complex represents a final signal to CARD9 [86]. Following endosomal detection by Dectin-1 of fungal pattern recognition molecules via Src/syc, CARD9 becomes activated via BCL10/MALT1 and converges on IKK to transcribe NFkβ [87]. The relationship between CARD9 and GM-CSF is typified by a case report of a 41-year old male with spontaneous CARD9 deficiency that

developed central nervous system candidiasis and was successfully treated with sargramostim [88]. Subtleties in genetic deficiencies exhibited by different CARD9 deficient patients are suspected to influence the outcomes of GM-CSF therapy in these patients. Clearly, more intensive work is required to determine the mechanism(s) that influence the interactions between GM-CSF and downstream signaling pathways affecting fungal infection in CARD9 deficient patients [89,90].

The anti-apoptotic signals via PI3K/AKT converge on both GM-CSF and BTK. The cardinal importance of these signaling factors is demonstrated by the reported increase of incidence of fungal infections following treatment with ibrutinib [91]. Tyrosine-protein kinase Tec and Btk are required for proper expression of GM-CSFRα in macrophages but not in dendritic cells, implicating Tec kinases in the lineage-specific regulation of GM-CSFRα expression [92].

BTK also activates PLCγ that in-turn increases intracellular calcium by opening calcium channels on the cell membrane and in the endoplasmic reticulum. Calcium signaling activates calcineurin, and results in NFAT translocation to the nucleus. This interrelation may explain the correlated degranulation and phagocytosis recorded in granulocytes during inflammation in the presence of GM-CSF [87]. In fact, BTK signals also activate RHO, WASP and actin, all related to cytoskeletal remodeling. Interestingly, BTK is also activated by TLR9; a pattern recognition receptor that detects fungal CpG DNA in the acidified endosomes [93]. Thus, GM-CSF increases phagocytosis via FcRγ and potentiates the BTK-calcineurin-CARD9 anti-fungal mechanism(s). Furthermore, Syk, a tyrosine kinase that activates CARD9, also increases GM-CSF biosynthesis [86].

Vora and colleagues have demonstrated that voriconazole combined with monocytes treated with sargramostim showed enhanced activity against *Aspergillus fumigatus*. [94]. Indeed, murine studies suggest that combination therapy using GM-CSF with traditional antifungal therapies can be effective for clearing *Aspergillus* infections.

Using a murine model in which the β chain of the GM-CSF receptor was deleted (GM-CSFRβ$^{-/-}$), these authors demonstrated that *A. fumigatus*-challenged animals had decreased rates of survival compared to wild type controls. The authors demonstrate that depletion of the β chain of the GM-CSFR reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activity, a potential mechanism for the lethality of the fungal infection.

Interestingly, these authors further demonstrated that in the WT mice, neutrophil NADPH oxidase activity could be augmented by administration of recombinant GM-CSF, suggesting a potential for GM-CSF as an inhibitory treatment for fungal infections [95]. However, this potential activity for GM-CSF with or without other combined fungal treatments needs to be examined in well-controlled human clinical trials designed to assess optimal dosing for recombinant GM-CSF as well as for optimal antifungal therapy. Thus, more research is necessary to refine the use of combination therapies, involving GM-CSF with traditional antifungal therapy, and to assess the potential optimal concentrations necessary for effective clearance with minimal toxicity.

Dimorphic Fungi:

Thermally dimorphic endemic fungi infect humans with intact as well as immunocompromised defenses. Examples that these fungi may infect people with intact immune defense systems (i.e., immune-competent) include fungi that cause histoplasmosis (e.g., *Histoplasma capsulatum*) and coccidioidomycosis (e.g., *Coccidioides immitis* and *Coccidioides posadasii*). In contrast, in immunocompromised individuals (e.g., HIV+ individuals or people on high levels of immunosuppressants, such as solid organ transplant patients), yeast such as *Blastomyces dermatitidis* or *H. capsulatum* pose a high risk for infection. Non-thermal dimorphic fungi (e.g., *Malassezia furfur*) may also lead to human infection, but are generally considered less lethal.

In the case of histoplasmosis, *H. capsulatum* are taken up by alveolar macrophages in the lung and are able to replicate within the macrophage phagolysosome allowing for an evasive mechanism [96]. In an effort to halt intracellular growth, infected macrophages upregulate GM-CSF and GM-CSFR which signal through JAK/STAT signaling causing limited intracellular zinc through sequestration via metallothioneins (MT) and enhanced superoxide production in order to kill fungi within the phagolysosome [97,98]. Taken together, GM-CSF-mediated changes in macrophage zinc homeostasis seem to serve a triple function in host defense: (1) starving the pathogen of an essential nutrients, (2) augmenting ROS production, and (3) shifting the balance of redox tolerance in favor of the macrophage [99]. In contrast, IL-4 signaling can reverse this effect (**Figure 4**).

Invasive Fungal Infections:

Invasive fungal infections (IFIs) can arise either from genetic mutation (e.g., CARD9) or immunotherapeutic induced response. The latter have increased proportionally with the advent of new immunotherapeutic interventions that suppress the host immune system. The source of IFIs are generally fungal spores that are inhaled (e.g., aspergillosis, histoplasmosis) or are absorbed through the skin (e.g., dermatophytosis) or through penetration into the mucosa by commensal organisms such as *Candida albicans*, as well as the ingestion of a toxin in contaminated food or drink (gastrointestinal disease).

Fungal infections can compromise immunocompetent and immunocompromised individuals. Examples of fungal infections of healthy individuals include vaginal infections, sinus infections and pneumonia. In immunocompromised hosts, *Aspergillus*, *Candida* and *Zygomycetes* pose major threats. Infections with *Zygomycetes* causing mucormycosis have been reported and managed with sargramostim. Infections with these three organisms in such individuals are usually life-threatening.

With the potential of a life threatening infection, it has recently been suggested for physicians taking care of CARD9-deficient patients with Candida species–related meningoencephalitis, it is important to: (1) aggressively institute intensive antifungal treatment early to avoid infection-related structural brain abnormalities that require shunt placement and (2) to use adjunct GM-CSF with the understanding that different patients can exhibit differential responses to GM-CSF treatment, including awareness for the potential of promoting eosinophil-mediated CNS immunopathology, particularly in patients with high burden CNS fungal infection and/or infection-related structural brain abnormalities (hydrocephalus and trapped ventricles) at treatment onset [90].

Interestingly, *Candida* infections may also induce GM-CSF production following contact with the host tissue, although there are apparent differences in the capacity of different species to induce GM-CSF. For example, co-culture of oral *Candida glabrata* with primary oral epithelial cells, or oral cell lines, induced increased levels of GM-CSF, as compared to co-culture with *C. albicans* [100]. Follow up experiments by the same authors demonstrated that the increase in GM-CSF was mediated through activation of NF-κβ and the stimulation was dependent upon adhesion of live *Candida* to the epithelial cells and could be enhanced by endocytosis of the candida [101]. The upregulation of GM-CSF may partially explain the respective levels of pathogenicity between *Candida* glabrata and *C. albicans*.

GM-CSF can alter the course of fungal infections by activating neutrophils as well as monocytes which may, in turn, provide local control of invasive fungi. GM-CSF production by NK cells is crucial to boost the candidacidal potential of neutrophils [44]. In a murine model Whitney and associates demonstrated that DCs, through Syk signaling, coordinate the entire innate immune control to systemic *C. albicans* as it provides IL-23p19 to NK cells that allows for production of GM-CSF, which in turn maintains the microbicidal activity of neutrophils, the main candidacidal effectors. Disruption of this cellular relay in

CD11cDSyk or IL-23p19 knock out mice causes susceptibility to systemic candidiasis and restoration of resistance can be achieved with GM-CSF treatment [102].

Conclusions:

GM-CSF-related pathways are of pivotal importance for immune response mechanisms and host defense. Numerous cell types can produce, as well as respond to GM-CSF. Given the pleiotropic nature of GM-CSF, more detailed studies are warranted to examine the fine detail of the complicated and often redundant interactions mediated by this important and complex modulatory cytokine. In the clinical setting factors including the duration and severity of infection, potential differences in strain virulence, and host immunologic status must be examined in greater detail.

In laboratory testing, sporicidal and improved hydrogen peroxide disinfectants were highly effective against *C. auris*, *C. glabrata*, and *C. albicans* for surface disinfection [103]. Because there seems to be increasing ability of fungi to develop resistance to antifungal therapies, new approaches to limiting or eliminating these infections by augmenting host response are desperately needed. Questions such as these raise the possibility that therapies such as sargramostim augmentation may help to establish immune competence and thereby facilitate the management of chronic intracellular infections. This protective role may improve patient's outcomes by limiting cellular destruction, for example, in the lung following invasive viral infections.

Given the newly developed antifungal therapies on the market, and the renewed interest in augmenting the host immune response through cytokine treatment and cellular immune therapy, the question becomes-where is immune augmentation likely to have the highest impact on fungal threats? A deeper insight into the utility of Sargramostim propelled by GM-CSF biology to stimulate innate immune system, when combined with anti-infective

agents may usher novel approaches to managing immunocompromised patients that are highly susceptible to these devastating infections.

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Disclosures

Conflicts of interest: In compliance with the ICMJE uniform disclosure form, all authors declare the following: Financial relationships: Luis O. Leal declares employment from Partner Therapeutics who manufactures and commercializes sargramostim. Other relationships: All other authors have declared that there are no other relationships or activities that could appear to have influenced the submitted work.

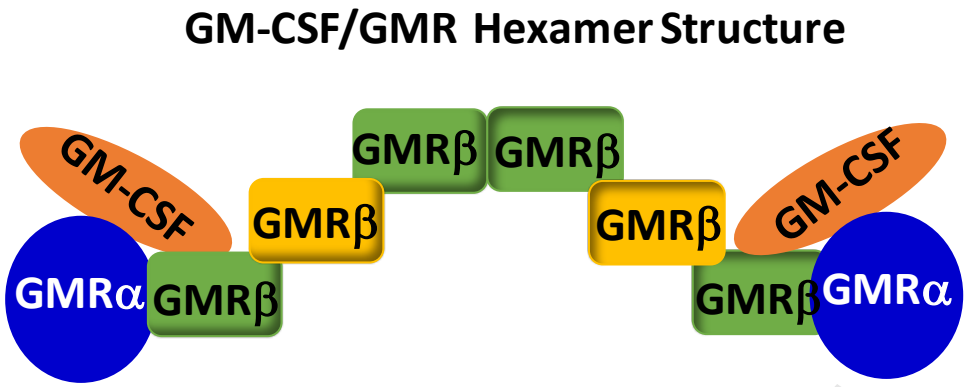

Figure 1 Graphic presentation of The GM-CSF/GMCSFR receptor-ligand complex
GM-CSF binds to the GMRα and the GMRβ complex to form a high affinity hexamer complex that then form dodecamer complexes by aggregation of the hexamer subunits.

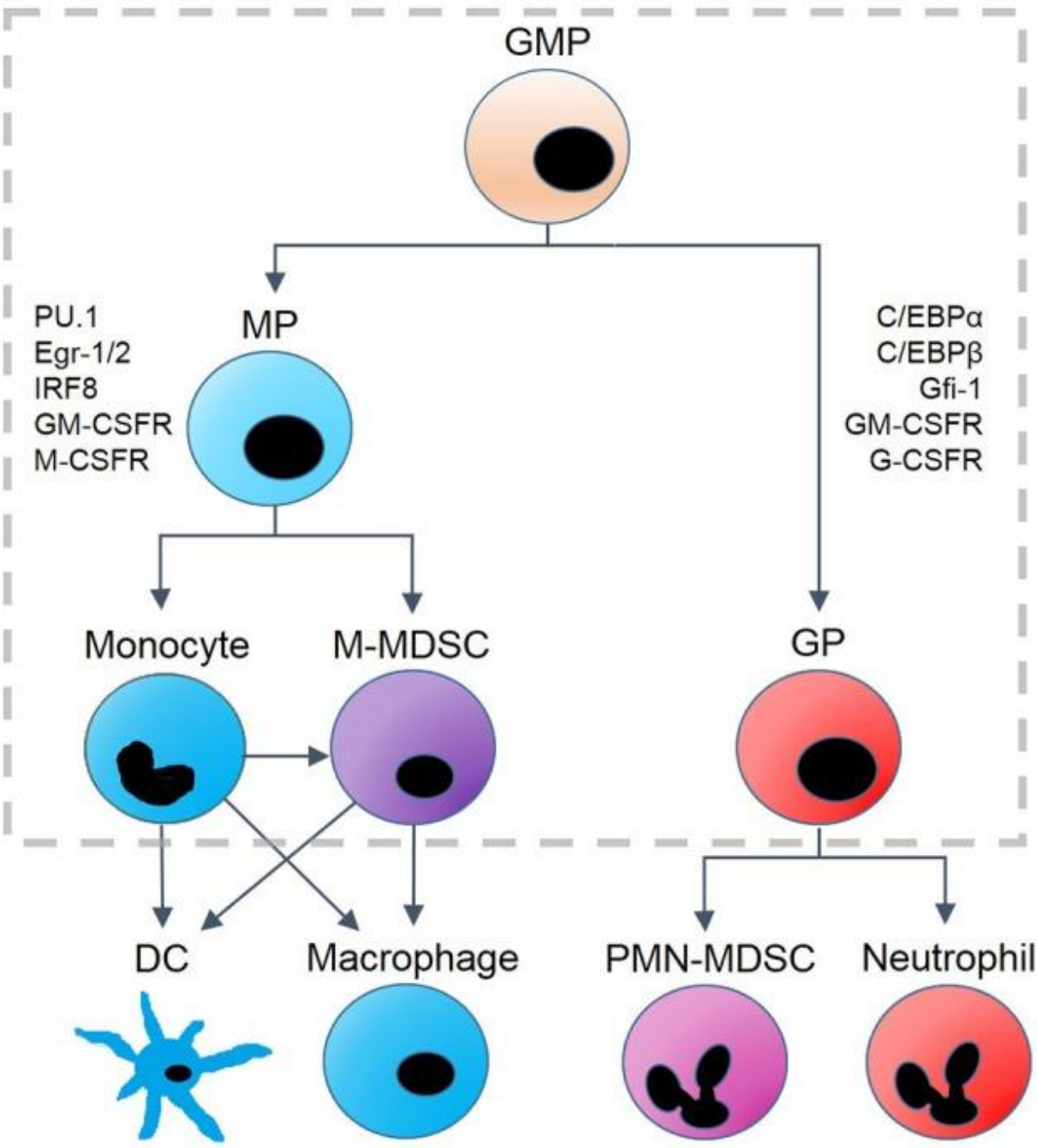

Figure 2. Hematopoetic lineage adapted from Weston et. al., 2018 [6]

Granulocyte-monocyte progenitors (GMPs) give rise to monocytic and granulocytic progenitors and their various progeny. Following differentiation of GMPs to either granulocyte or monocyte progenitors (GP or MPs), distinct patterns of protein expression result in conversion of precursor populations to monocyte and monocyte-derived suppressor cells (M-MDSCs) via PU.1, Egr-1/2, IRF8, M-CSF and GM-CSF or GP via upregulation of C/EBPα, C/EBPβ, Gfi-1, G-CSFR and GM-CSF. Monocytes then differentiate further into dendritic cells (DCs) and macrophages whereas GPs account for the polymorphonuclear (PMN) MDSCs and neutrophils. The model presented by Weston and colleagues captures the conversion of the precursor cells via GM-CSF interaction with other stimulatory growth factors

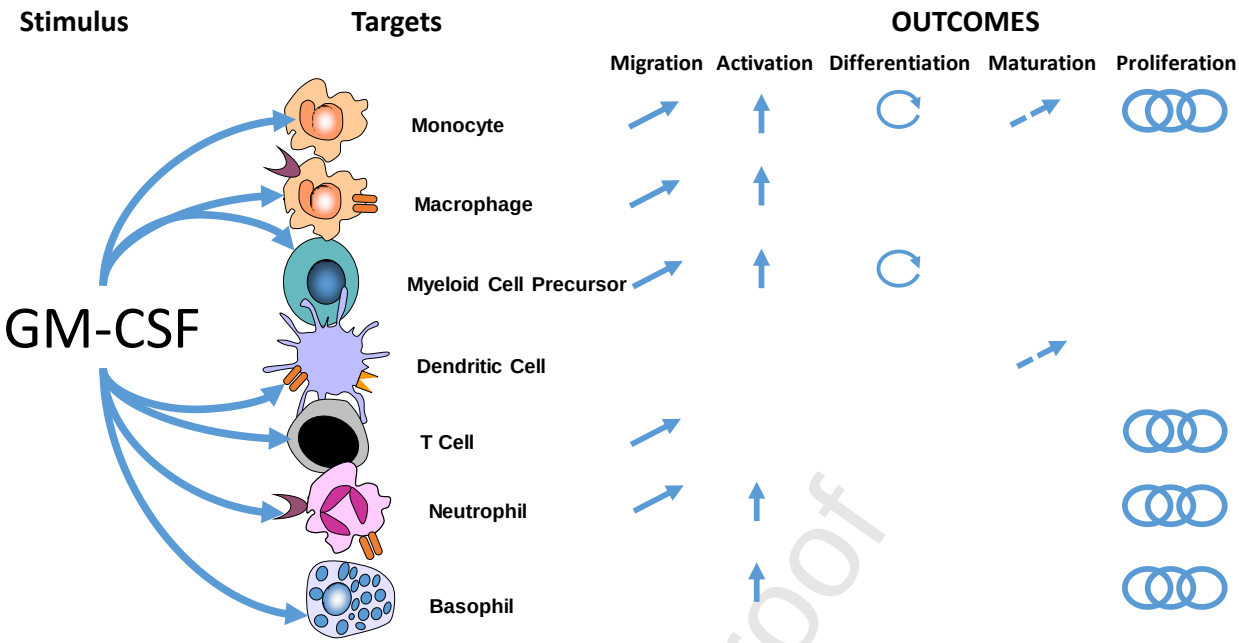

Figure 3. GM-CSF elicits immune cell responses
GM-CSF has pleiotropic effects on host immune cells ranging from attracting these cells, causing activation and proliferation of these cells as well as differentiation and maturation. The indicated outcomes for each GM-CSF-Immune cell interaction is indicated on the right side of the diagram. All of these indicated outcomes are also contextually confined depending upon the location of the interactions.

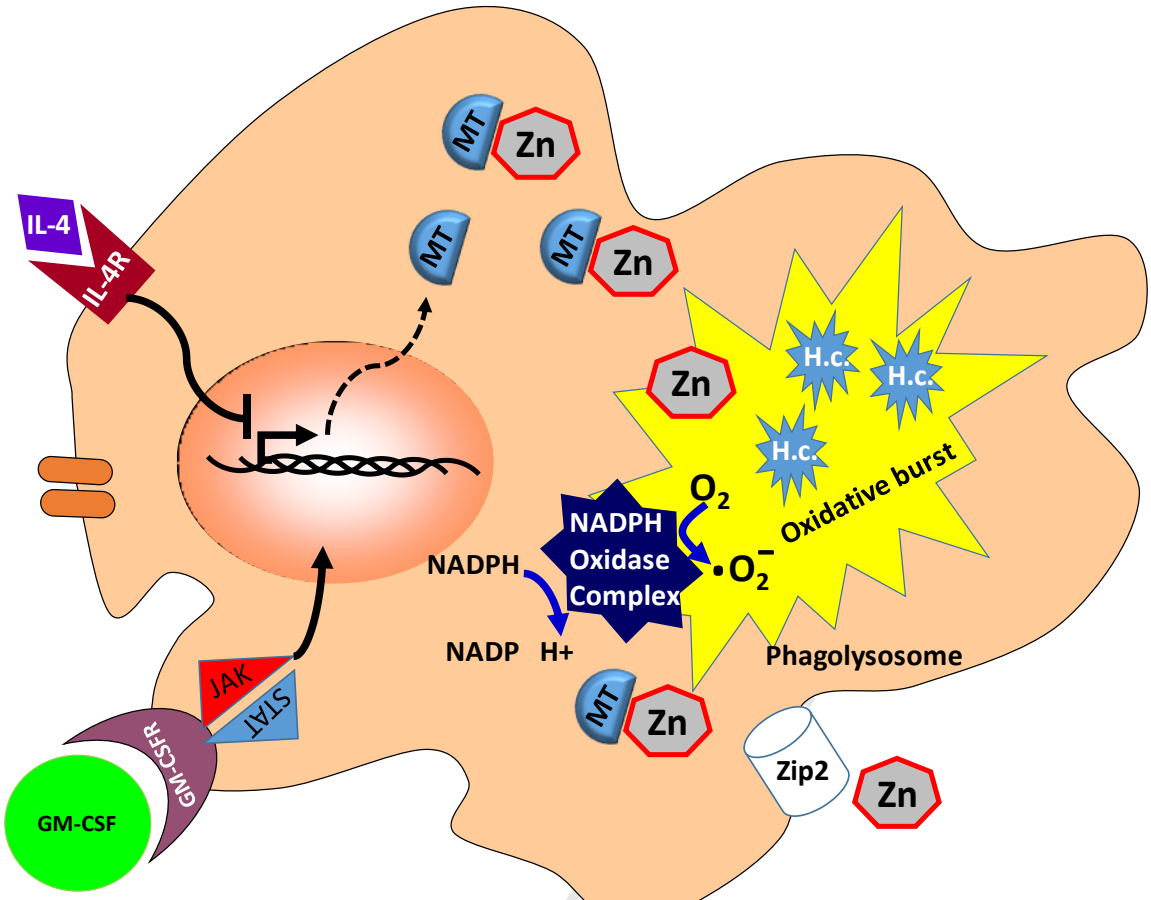

Figure 4. Macrophage fungal killing Adapted from Crawford et. al., 2015 [98]

Infected macrophages (e.g., *Histoplasma capsulatum*, H.c.) upregulate GM-CSF which signals through JAK/STAT pathways, which is inhibitable by IL-4 signaling. Metallothioneins sequester Zinc (Zn) resulting in increased superoxide burst and accelerated fungal killing in the phagolysosome. Thus, GM-CSF enhances intracellular killing of fungal pathogens.

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- GM-CSF-related pathways are important for immune response mechanisms
- Immunomodulatory properties of GM-CSF target tissues that express GM-CSFRα
- GM-CSF plays a pivotal role during bacterial infections
- The role of GM-CSF in fungal infection is complex.
- Combination GM-CSF therapy with traditional antifungal therapies can be effective